



Sveriges lantbruksuniversitet  
Swedish University of Agricultural Sciences

Department of Molecular Biology

# **Proteomics studies of neurotoxic amyloid $\beta$ oligomers using size exclusion chromatography**

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# Proteomics studies of neurotoxic amyloid $\beta$ oligomers using size exclusion chromatography

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## **Abstract:**

The prime toxic species that causes Alzheimer's disease is believed to be oligomeric aggregates of the amyloid  $\beta$  peptide ( $A\beta$ ). The major aim of the work was to develop methods to study which proteins in human plasma that interacts with oligomeric neurotoxic forms of  $A\beta$ . The interactions of  $A\beta$  with human biological fluid proteins are important for drug discovery efforts against Alzheimer's disease but still not very well explored. Stable peptide oligomers were formed by a special variant of  $A\beta$  (called  $A\beta_{42cc}$ ). The co-elution of  $A\beta_{42cc}$  oligomers with human blood serum was carried out using size exclusion chromatography (SEC). The eluted fractions were analyzed by SDS-PAGE but no strong interaction between  $A\beta$  oligomers and blood serum proteins could be observed.

**Key words:** *Alzheimer's disease, proteomics, amyloid- $\beta$  oligomers, size exclusion chromatography.*

## Popular Science Summary

The most common neurodegenerative disorder of aging is Alzheimer's disease. Alzheimer's disease is characterized by the large extracellular deposits of senile plaques in the brain that consists of aggregated fibrillar amyloid  $\beta$  peptides. Amyloid  $\beta$  peptides are 39-43 amino acids in length and produced by the cleavage of amyloid precursor protein (APP) by  $\gamma$ -secretase and  $\beta$ -secretase enzymes. The biochemistry of amyloid  $\beta$  oligomers has been a fascinating area of research for therapeutic inventions. The aim of my thesis was to develop methods to study which proteins in human blood serum that interacts with oligomeric neurotoxic forms of the amyloid  $\beta$  peptide. One part of my thesis was the production of recombinant A $\beta$ 42cc in *E.coli* and purification of A $\beta$  oligomers using separation techniques. In the next step, the thesis focused on developing methods to capture binding proteins from human blood serum using A $\beta$ 42cc oligomers. For this purpose, co-elution of A $\beta$ 42cc oligomers with human blood serum was investigated using size exclusion chromatography as a method for interaction studies of human blood serum proteins with A $\beta$ 42cc oligomers. We know in SEC large size molecules elute earlier than the smaller size molecules. It was expected that the proteins that interacts with A $\beta$ 42cc oligomers will elute earlier than the non-interacting proteins. Those fractions were further analyzed by using SDS-PAGE to separate different components. From the gel it was observed that there was no strong interaction of A $\beta$ 42cc oligomers with human serum proteins.

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**Aim:**

The aim of this study is to develop methods to study which proteins in human plasma that interacts with oligomeric neurotoxic forms of the amyloid  $\beta$  ( $A\beta$ ) peptide

**It investigates:**

- Size exclusion chromatography of the mixtures of special variant of  $A\beta$  ( $A\beta_{cc}$ ) oligomers and plasma.
- Analysis of the eluted fractions by gel electrophoresis (SDS-PAGE).



## Introduction:

Alzheimer's disease is an age-related progressive degenerative disorder. Alzheimer's disease is characterized by the loss of neurons and synapses from the brain and accumulation of senile plaques and neurofibrillary tangles (1). Amyloid  $\beta$  peptide ( $A\beta$ ) is a 39-43 amino acid residues long cleavage product of amyloid precursor protein (APP) and is the main component of senile plaques. APP is a single-pass trans-membrane protein with 695-770 amino acids, transported from endoplasmic reticulum to the cell surface through a secretory pathway (2) and expressed at high levels in the brain and metabolized in a rapid and highly complex fashion by a series of sequential proteases. APP is cleaved by membrane associated  $\gamma$  and  $\beta$ -secretases which generates peptides with different sizes like  $A\beta(1-40)$  and  $A\beta(1-42)$ .  $A\beta(1-42)$  has increased hydrophobicity and tendency to aggregate so, it is considered more neurotoxic than  $A\beta(1-40)$  (3). Synthetic  $A\beta$  spontaneously aggregates into  $\beta$ -sheet rich fibrils. The insoluble fibrillar aggregates were reported to be neurotoxic *in vivo* and *in vitro* so that it was believed that fibrils are responsible for neurodegeneration in Alzheimer's disease (4).

In some studies ranges of the assemblies, from dimers to 24 mers or even higher molecular weights, of  $A\beta$  are reported (5, 6, and 7). Although some studies strongly supports that such soluble  $A\beta$  oligomers are the causative agent of Alzheimer's disease, the biological and structural characteristics of  $A\beta$  oligomers and their formation mechanism is still unclear.

Alzheimer's disease is still incurable and the mortality rate is increasing rapidly. It is very important to learn more about the molecular mechanism of Alzheimer's disease to treat and prevent Alzheimer's disease in the future. To develop effective therapeutics based on interactions between  $A\beta$  and human blood serum proteins, increased understanding of the  $A\beta$  binding ligands is necessary.

## History of Alzheimer's disease:

In 1901, a patient named Auguste D, 51 years of age was suffering from cognitive and language deficits, auditory hallucinations, delusions, paranoia aggressive behavior. Her condition was studied by a doctor, Alois Alzheimer. In 1906 Auguste died. In November of the same year Alzheimer presented Auguste's case at a psychiatry meeting and published his talk in 1907. Today this degenerative brain disease bears his name.

Among of the two distinct pathologies one is neurofibrillary tangles that was summarized by Alzheimer as intracellular aggregates later it was observed that it composed of hyperphosphorylated and cleaved form of the microtubule associated protein *tau*. Another one is miliary foci. The miliary foci were a dystrophic neuronal process that surrounds a special substance in the cortex (8). After isolation and purification it was confirmed that special substance was a 4.2 kDa protein and 40-42 amino acids long and it is a cleavage product of long precursor (9). Their prediction was verified by cloning APP in 1987 (10). The peptide that was isolated by Glenner and Wong is known as A $\beta$  peptide.

In 2001, the first Alzheimer disease vaccine trial was conducted in U.S. and Europe. There were serious side effects and it was stopped later. In 2002, a new drug called NMDA receptor blocker was developed. Some currently used treatments include cholinergic pathway of degeneration, anti-oxidants, anti-inflammatory drugs and estrogen.

AD is still incurable and Alzheimer's disease today affecting more than 26 million peoples all over the world with projections of a fourfold increase by 2050 (11).

### **Pathology of Alzheimer's disease:**

In Alzheimer's disease, A $\beta$  that is expressed normally and ubiquitously throughout the life, forms fibrils that deposits in the brain as senile plaques. This senile plaques and neurofibrillary tangles, which are built by hyper phosphorylated tau protein, are the pathological hallmarks of Alzheimer's disease (12 and 13).

The power house of cell is known as mitochondria that meets the energy demand of the cell. In case of some defects in mitochondria it cannot supply energy to the cell but it can damage the cell by producing reactive oxygen species (ROS), which leads to cellular oxidative stress (14). A $\beta$  can induce the generation of ROS and production of apoptogenic proteins and thus can promote the toxicity to neurons (15, 16).

In neurons, the microtubule sensitizing protein is *tau*. An inflammatory response can be triggered by hyperphosphorylated *tau* proteins. Hyperphosphorylated *tau* proteins can disrupt the normal microtubule binding which leads to the loss of axons and defective neuronal communication. Later this can lead to the neuronal loss (17, 18).

For the nucleation of A $\beta$  fibrils two mechanisms have been proposed. The first one involves the self-assembly of the A $\beta$  monomers that undergoes a conformational change to become the

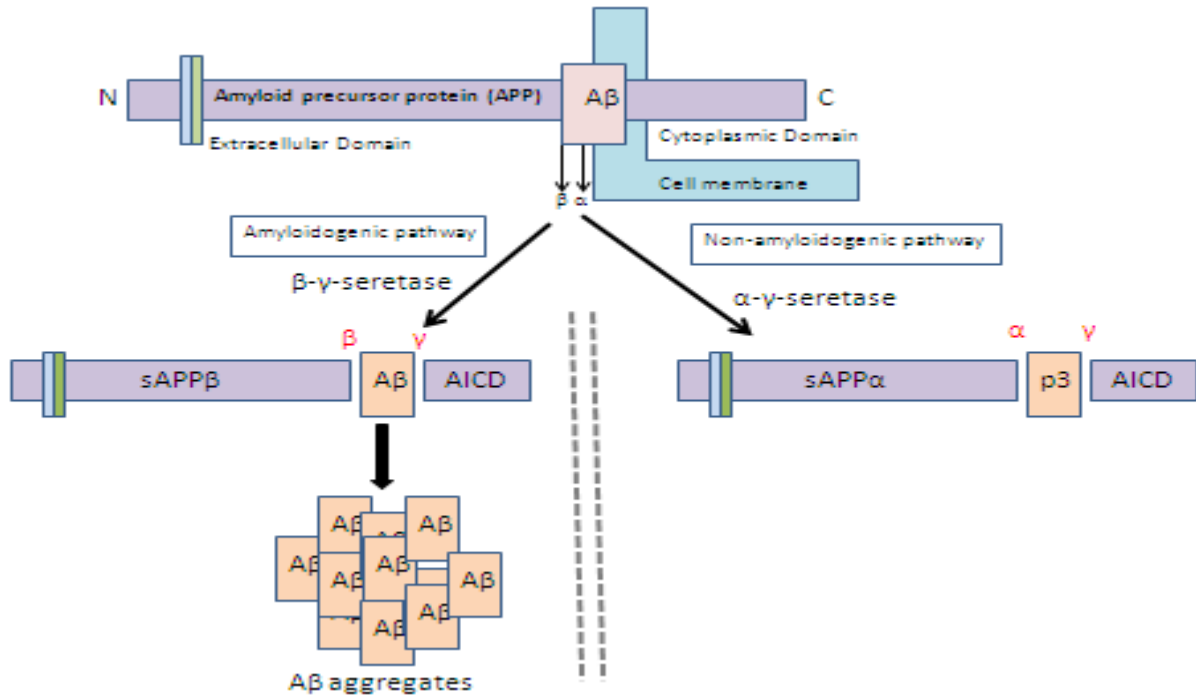
fibril nucleus where the second one involves the alternative pathway of heterogeneous nucleation that results from the outgrowth of fibrils from A $\beta$  seeds (19).

A heated debate for the last 20 years has been ongoing whether *tau* protein or A $\beta$  is the initial event in Alzheimer's disease. As this thesis is mainly about A $\beta$  oligomers so, from now only A $\beta$  will be focused on.

### **Processing of APP:**

APP is strongly linked with the development of Alzheimer's disease. The APP gene located on chromosome 21. APP is proteolytically cleaved by alpha ( $\alpha$ ), beta ( $\beta$ ) and gamma ( $\gamma$ ) secretases (20). The proteolytic processing of APP can be either via amyloidogenic or non-amyloidogenic pathway (shown in fig 1).

The non amyloidogenic pathway involves the sequential cleavage by  $\alpha$ -secretase within the amyloid peptide domain that is later cleaved by  $\gamma$ -secretase and produces secretory APP (sAPP)  $\alpha$  segment and the membrane bound C-  $\alpha$ -terminal fragment ( $\alpha$ -CTF) is cleaved by  $\gamma$ -secretase and produces P3, a truncated non-amyloidogenic peptide. On the other hand the amyloidogenic pathway involves the cleavage by  $\beta$ -secretase at the C-terminus and releases sAPP  $\beta$  and a membrane bound C $\beta$ -terminal fragment ( $\beta$ -CTF). Later the multimeric  $\gamma$ -secretase cleaves  $\beta$ -CTF at different cleavage sites producing amyloidogenic A $\beta$  peptides of different sizes. The main variants are A $\beta$ (1-40) and A $\beta$ (1-42) containing 40 and 42 amino acids respectively.



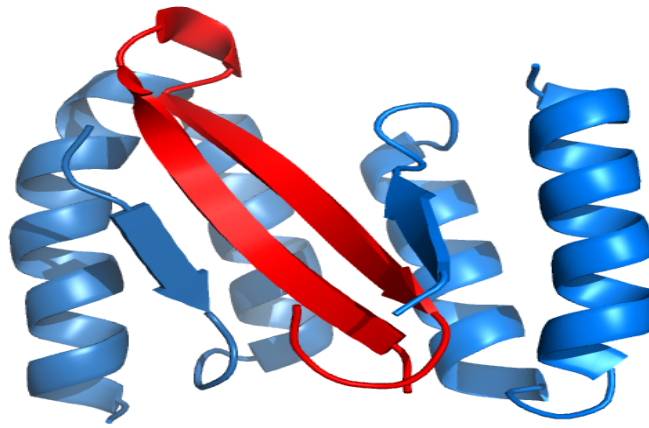
**Fig.1: Processing of APP.**

#### **Aβ42cc- Z<sub>Aβ3</sub> complex:**

Aβ42cc is an engineered variant of Aβ(1-42) that enables the isolation of stable oligomers. Ala21 and Ala30 used for the purpose of protein engineering, as their β-carbons are located on opposite β-strands at a distance of 4.2 Å from each other. A Cys21/30 disulfide accommodated with favorable conformational energy and without perturbing the hairpin structure where the 12 backbone hydrogen bond is predicted to form in Aβcc (21).

It can be produced in *E.coli* cells by co-expression with Z<sub>Aβ3</sub> affibody. Z<sub>Aβ3</sub> co-expression also permits the recombinant production of Aβ (1-42) (3).

Z<sub>Aβ3</sub> is a di-sulfide-linked homodimer of affibody subunits (22). Affibody Z<sub>Aβ3</sub> binds to the central C-terminal part of the Aβ (fig 2) that then adopts a β-hairpin conformation.



**Fig.2: Structural representation of A $\beta$ -Z $_{A\beta 3}$  complex.** Here the red colored structure represents the A $\beta$  peptide and the blue colored structure represents Z $_{A\beta 3}$  affibody. (Fig source: PDB accession no: 20TK, (22), kindly provided by Christofer Lendel).

### **Blood serum:**

In blood, the serum contains all proteins that are not involved in blood clotting (coagulation) and all the electrolytes, hormones and any exogenous substances. In clinical testing serum is the preferred specimen. Blood proteins are known as plasma proteins or serum proteins. Blood proteins have several functions including circulation of transport molecules for lipids, hormones, vitamins and metals, regulation of cellular activity and functioning and in the immune system. Blood contains about 3.5-5.0 g/dl albumin (normal level), 60% of total volume. Immunoglobulin contributes about 18% (1.0-1.5 g/dl, normal level), fibrinogen contributes about 4% (0.2-0.45 g/dl) and other regulatory proteins contains <1% of total volume. Other types of blood proteins include prealbumin alpha1 antitrypsin, alpha 1 acid glycoprotein, alpha 1 fetoprotein, haptoglobin, alpha 2 macroglobulin, ferroplasmin transferring C3/C4 beta 2 micro globulin, beta lipoprotein, gamma globulin proteins, C-reactive protein (CRP).

The understanding of human serum protein interaction with A $\beta$  oligomers is very important for novel therapeutic inventions. As a part of development of methods to identify the serum protein interaction with A $\beta$  oligomers, I have introduced size exclusion chromatography (SEC) to separate the proteins according to their sizes where the proteins interacting with the large A $\beta$  oligomers proteins were expected to elute earlier than the non-interacting proteins.

This thesis report will discuss SEC as a method to capture binding proteins from biological fluids (blood serum) using A $\beta$  cc oligomers.

### **Techniques used in this project:**

#### **Immobilized metal ion affinity chromatography :**

Immobilized metal ion affinity chromatography (IMAC) is based on the binding between proteins and metal ions. Because of different amino acid compositions, proteins have different affinities towards different metal ions. Proteins can also be engineered to contain a poly-histidine tail that can act as a ligand towards metal ions.

A mixture of proteins can be separated according to their interacting abilities towards metal ions. Proteins with more histidine residues bind more tightly than those proteins with fewer histidine residues. Several types of metal ion affinity chromatography techniques (for example, Co, Cd, Fe, Ni, Zn) have been developed to separate proteins.

#### **Size exclusion Chromatography:**

In SEC the molecules are separated based on size (23). It is also called molecular exclusion or gel permeation chromatography. When an aqueous solution is used to transport the sample through the column then it is called gel-filtration chromatography. Protein mixtures are applied to a gel filtration column containing chromatographic matrix where stationary phase consists of porous beads with a defined set of pore sizes. Proteins are said to be included if they are small enough to fit inside the pore of beads and have the access to both the stationary inside the beads and the mobile phase between beads and elute last in a gel filtration separation. Proteins are said to be excluded if they are too large to fit inside of any pore. Those larger proteins only have the access to the mobile phase and elute first. Proteins with intermediate size partially can fit inside but not in all of the pores of beads and elute between the large (excluded) and small (totally included) proteins. The small proteins that enters inside

the beads elute at last. One of the Advantages of this method is that it separates large molecules from the small molecules with a minimal volume of elute (24).

The main application of the gel-filtration chromatography is the fractionation of the proteins and other water soluble polymers. As the sample do not interact with the stationery phase there is no sample loss. This technique can be combined with other techniques to further separate molecules according to other characteristics like acidity, basicity, charge and affinity for certain compounds.

The technique was invented by Grant Henry Lathe and Colin R Ruthven, working at Queen Charlotte's hospital, London (25, 26). Ruthven and Lathe introduced starch gels as the matrix, Jerker Porath and Per Flodin later introduced dextran gels (27). Other gels with size fractionation properties including agarose and polyacrylamide. In 1964, J.C. Moore of the Dow chemical company published his work on the preparation of gel permeation chromatography columns based on cross-linked polystyrene with controlled pore size (28).

To perform a separation gel filtration medium is packed into a column to form a packed bed. The medium is the porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability and inertness. The packed beds are equilibrated by using buffer. Buffer fills the pores and the space between the matrix particles. The particles of different sizes will elute through a stationery phase at different rates. All the particles are loaded simultaneously or near-simultaneously, particles of the same size should elute together.

The apparatus that is used for the separation of molecules in SEC is called column. A SEC column consists of hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes. To maximize the resolution the proper column packing is very important. An over packed column can collapse the pores in the beds and thus results the loss of resolution. An under packed column can reduce the relative surface area of the stationery phase that are accessible to smaller species and results those species spending less time trapped in pores.

The eluent is collected in constant volumes that are known as fractions. The collected fractions are then examined by spectroscopic techniques to determine the concentration of the particles eluted. Refractive index and ultraviolet absorbance are commonly used to examine eluates.

## **Experimental parameters of size exclusion chromatography:**

### **Sample:**

The viscosity of sample should not be so large to cause hydrodynamic instability. The pH, ionic strength and composition are not significant as long as they do not affect the sizes or the stability of the molecules to be separated.

### **Column:**

The way of packing the column is the most important characteristic of gel filtration column. In evenly packed column, the sample zone is not unnecessarily broadened when it passes down the column and good results are obtained. Good results cannot be obtained with unevenly packed column. The length of the column is significant because it affects both the resolution and the elution time. Resolution is proportional to the square root of column length and elution time is proportional to column length.

### **Running conditions:**

The rate of eluent flow through affects both the speed at which the separation is obtained and the resolution which can be achieved. Lower flow rate gives better resolution at least for large molecules (29). In the form of an elution diagram the results of gel filtration are expressed. It shows the variation of solute concentration in the eluate with the volume of eluent passed through the column. Continuous detection using a UV monitor gives an immediate permanent record, a chromatogram when working with protein and nucleic acid. From this diagram the elution volume ( $V_e$ ) can be defined.

### **SDS-PAGE:**

Electrophoresis is a technique where the macromolecules are separated by applying an electric field. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is a commonly used method for separating proteins where the polyacrylamide gel is used as a support medium and SDS is used to denature the proteins. SDS is an anionic detergent that gives the molecules a negative charge over a wide pH range. The amounts of SDS that bind to the polypeptide chains proportionally to its relative molecular mass when the complex protein structures are destroyed by SDS. These structures are attracted towards the anode. Because of the presence of SDS, proteins are separated according to their sizes.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Relative molecular masses, relative abundance of major protein in a sample can be estimated



by SDS-PAGE. Moreover it is also possible to monitor the purity of protein, fractionation progress and the efficiency of the purification procedure by SDS-PAGE.

### **Silver staining:**

Silver staining is a procedure of detecting proteins after electrophoretic separation on polyacrylamide gels. It provides excellent sensitivity (in the low nanogram range) using very simple and cheap equipment and chemicals. Silver staining is compatible with downstream processing such as mass spectrometry analysis after protein digestion. The sequential phases of silver staining are protein fixation, then sensitization, then silver impregnation and finally image development. Switzer et al., introduced silver staining in 1979 (30). Silver staining provides a very sensitive tool for protein visualization with a detection level of 0.3-10 ng (31).

The basic mechanism of silver staining is protein detection based on the binding of silver ions to the amino acid side chains, primarily the sulfhydryl and carboxyl groups of protein (31, 32, 33, 34), that is followed by reduction to free metallic silver (35, 36). Based on the reduction the protein bands are visualized as spots. The image of protein distribution in the gel is visualized as the difference in the oxidation-reduction potential between the gel's area occupied by the proteins and the free adjacent sides. Silver amine or alkaline methods, and silver nitrate or acidic methods are the two general silver staining protocols (37). The silver amine or alkaline methods usually have lower background and are more sensitive but the problem is that it requires longer procedures. On the other hand acidic procedures are faster and less sensitive than the alkaline methods. Several silver staining procedures have been developed with different advantages regarding timing, sensitivity, cost and compatibility with other analytical methods.

## **Materials and Methods:**

### **Expression:**

A $\beta$ 42cc-Z<sub>A $\beta$ 3</sub> was expressed in *Escherichia coli* origami B cells from plasmid pACYDUet-1 coding for A $\beta$ 42cc and the affibody Z<sub>A $\beta$ 3</sub> under separate promoters. In addition to Z<sub>A $\beta$ 3</sub> affibody sequence, the construct contained an N-terminal (His)<sub>6</sub> tag. Expression vector pACYCDuet is designed for the double cistronic coexpression of two target genes and contains two multiple cloning sites (MCS) and each of the MCS is preceded by a T7

promoter/lac operator and a ribosome binding site. Expression vector contained the elements in the following order: T7 promoter-1- MA $\beta$  - T7 promoter-2- (His)<sub>6</sub> z A $\beta$ 3- T7 terminator. Thus A $\beta$ cc was produced in *E.coli* by co-expression with Z<sub>A $\beta$ 3</sub> complex. The cells are resistant to chloramphenicol (14). Cells were grown in overnight pre-culture at 37 °C and 120 rpm agitation. Overnight culture contained TB medium, potassium phosphate buffer and antibiotics (34 mg/l chloramphenicol, 12.5 mg/l tetracycline and 15 mg/l kanamycin). 75 ml of overnight culture was added per 0.9 l TB together with the antibiotics (in case of 75 ml of 1X TB medium, 8.3 ml of 0.89 M (0.17 M potassium di-hydrogen phosphate and 0.72 M di-potassium hydrogen phosphate) potassium phosphate salt (10 X concentrate) and 83  $\mu$ l of each of three antibiotics (kanamycin 15 g/l, tetracycline 12.5 g /l, chloramphenicol 34 g /l) in a flask. Each 1 l of TB medium contains 12 g tryptone, 24 g yeast extract, and 4 ml glycerol in water solution. The main culture was incubated at 37 °C with agitation (120 rpm). The OD value at 600 nm was monitored using UV spectrophotometer (Shimadzu). Protein expression was induced at OD(600 nm)=0.6-0.8 with 75  $\mu$ l of 1 M IPTG (isopropyl  $\beta$ -D-1-thiogalactopyranoside). IPTG is a mimic of lactose and will induce the expression of genes under the control of lac Z promoter. Cells were incubated for 4 hours after induction with IPTG. Cells were harvested by 20 min centrifugation at 4500 rpm at 4 °C. The cell pellet from 1 l culture was resuspended in 30 ml 100 mM tris-hydrochloride; 500 mM sodium chloride plus 0.25-0.5 tablet of complete protease inhibitors cocktail (Roche). The resuspended cells were transferred into a 50 ml conical bottom falcon tube and kept at -20 °C.

### **Cell lysis:**

Cell pellets were completely thawed and kept on ice. The sample was sonicated on ice bath (ice plus some water for good heat transfer). It was sonicated using Vibra cell T.M. (CiAB Chemical instruments AB) at 75% amplitude in pulses (5 s ON and 9.9 s OFF) with a total sonication time of 2.5 min. Insoluble materials were removed by centrifugation of the cell lysates for 40 min at 18000 rpm at 4 °C (Sorvall centrifuge, ss-34 rotor). The supernatant was filtered through 0.45  $\mu$ m and then 0.2  $\mu$ m filter.

### **IMAC:**

The first step of protein purification was achieved by IMAC employing an Äkta purifier. After recharging the IMAC column with 0.1 M nickel sulfate salt the filtered protein sample was loaded onto 5 ml Hi Trap IMAC HP Ni-NTA column (GE Healthcare). IMAC was carried out in 20 mM tris-hydrochloride, pH 8.0; 500 mM sodium chloride (buffer A) and 20

mM tris-hydrochloride, pH 8.0; 500 mM sodium chloride; 300 mM imidazole (buffer B). After loading the lysate, the column was washed with 4% buffer B for 3-4 min, then 100% of buffer B for 10 min to elute the complex. The fractions supposed to contain A $\beta$ 42cc-Z $_{A\beta 3}$  complex (3) were collected and pooled and EDTA was added to a final concentration of 5 mM. EDTA is used to prevent metal ion binding with the protein complex.

The IMAC fractions that are supposed to contain A $\beta$ 42cc-Z $_{A\beta 3}$  complex was concentrated on 5 kDa cut off vivaspin to OD=3.9 AU at 280 nm measured using Nanodrop 1000 spectrophotometer (Thermo scientific).

#### **SEC:**

The concentrated eluted fractions from IMAC were loaded in 1.5-2 ml samples onto Superdex 75 10/300 column equilibrated with 20 mM tris-hydrochloride, pH 8.0; 200 mM sodium chloride at 0.7-0.9 ml/min flow rate. After elution, the fractions containing A $\beta$ 42cc-Z $_{A\beta 3}$  (known elution volume as the purification was carried out according to an established protocol) were concentrated on 5 kDa cut-off vivaspin to OD=4.5 AU at 280 nm measured using Nanodrop 1000 spectrophotometer (Thermo scientific).

#### **Denatured IMAC:**

From SEC, 1-5 ml of concentrated sample was diluted in 25 ml of 7 M guanidinium hydrochloride (GdnHCl) (>99% pure sigma), 0.5 M sodium chloride, 0.1 M tris-hydrochloride; pH 8.0, and kept on a shaker at room temperature until the GdnHCl dissolved. The tube containing the sample was then kept on a titling table for 30 min and then the protein sample was filtered using 0.2  $\mu$ m filter. Then denatured IMAC was run. Sample was loaded at 0.5 ml/min onto a 5 ml Hi Trap IMAC HP Ni-NTA column (GE Healthcare) equilibrated with 20 mM tris-hydrochloride; pH 8.0; 7 M GdnHCl. Most of the A $\beta$ 42cc elutes with the flow through but some binds to the column (as judged by SDS-PAGE later). The flow through was collected and the column was washed with an imidazole gradient of 0-40 mM for 30 min, which was followed, by a gradient to 400 mM imidazole for 5 min then affibody Z $_{A\beta 3}$  containing fractions (as judged by SDS-PAGE later) elutes.

#### **SDS-PAGE:**

Fractions that are supposed to contain A $\beta$ 42cc and Z $_{A\beta 3}$  were collected and 100  $\mu$ l of sample from each fraction were dialyzed against 7 M urea overnight. Then the dialyzed fractions were prepared for SDS-PAGE; 10  $\mu$ l of loading buffer (containing 4 M urea, 15% glycerol,

10% SDS and 15 mM tris (2-carboxyethyl) phosphine) and 20 µl of sample were mixed. For further denaturation the peptides the sample was kept on 100 °C for 5 min. Then SDS-PAGE was run using 0.1 M tris as anode buffer and tricine as cathode buffer. About 15 µl of sample was loaded with Hamilton syringe. The syringe was washed with water between samples. Molecular size marker (5 µl) was loaded in one or two wells. The gel was run at 130-140 V until the blue bands reached the bottom of the gel. The gel was then removed and placed in fixation solution.

### **Silver staining:**

After the SDS-PAGE the gel was removed from the cassette and placed into a tray containing appropriate volume of fixation solution (50% ethanol, 12% acetic acid, 0.05% formalin). Fixation solution was added to restrict protein movement from the gel matrix. The gel was put on titling table for 1 hour. The fixation solution was discarded and the gel was washed with 20% ethanol for 20 min. The washing solution was changed 3 times during this time. The ethanol solution was discarded and sensitizing solution (0.02% (w/v) sodium thiosulphate) was added and incubated for 2 min. The sensitizing solution was discarded and the gel was washed with deionized water. After discarding the water, cold silver staining solution (0.2% (w/v) silver nitrate, 0.76% formalin), was added and the gel was kept in it for 20 min. After staining was done the gel was washed with deionized water for 60 s. The gel was rinsed with the developing solution. New portion of developing solution was added and the protein bands were developed by incubating the gel in 300 ml of developing solution (6% (w/v) sodium carbonate, 0.0004% (w/v) sodium thiosulfate, 0.05% formalin) for 2-5 min. The reduction reaction was stopped by adding 50 ml of terminating solution (12% acetic acid).

### **Production of A $\beta$ 42cc oligomers:**

A $\beta$  containing fractions from denatured IMAC were concentrated using 3 kDa cut-off to a final concentration of OD(280 nm)=1.3 AU. The sample was dialyzed against 20 mM sodium phosphate pH 7.2; 50 mM sodium chloride with 5 mM EDTA acid overnight.. EDTA has the ability to sequester metal ions. A $\beta$  oligomers formed spontaneously during dialysis against sodium phosphate at pH 7.2. Then the oligomeric solution was heated at 60 °C for 10 min in water bath and again dilysed to remove EDTA (same buffer). The peptide concentration was 0.49 mM. Finally the sample was frozen in liquid nitrogen and stored in -20 °C freezer.

**Blood serum:**

The blood serum (normal human serum, single donor) in our experiment was purchased from 3H biochemical AB, Uppsala, Sweden. Prior using in the experiments the serum sample was stored in -20°C.

**Columns used in this project:**

Sephacryl S-100 HR (GE Healthcare)

Superose 12HR 10/30 (GE Healthcare)

Superdex 200 (GE Healthcare)

Superdex 75 10/300 (GE Healthcare)

**Selection of buffer solution for size exclusion chromatography of A $\beta$  oligomers and blood serum:**

Three different buffer compositions like 20 mM sodium phosphate pH 7.4; 50 mM sodium chloride, 20 mM sodium phosphate pH 7.4; 100 mM sodium chloride and 20 mM sodium phosphate pH 7.4; 150 mM sodium chloride were used in Superdex 200 HR 10/300 gel filtration experiments with A $\beta$  oligomers and blood serum. High quality chemicals and water were used for the preparation of buffer and filtered through 0.2  $\mu$ M filter. Buffer solution was degassed as it was filtered under vacuum condition. A $\beta$ cc oligomeric sample (absorbance at 280 nm = 0.6 AU) and of blood serum sample (absorbance at 280 nm = 43.9 AU) were used for buffer test. 50  $\mu$ l of A $\beta$  oligomeric sample and 25  $\mu$ l blood serum was loaded on to Superdex 200 HR 10/300 gel filtration columns.

**Selection of gel filtration column with best resolution:****Resolution:**

The resolution is defined as the degree of separation between peaks of a gel filtration separation. The inevitable broadening of peaks during the chromatographic process can make it difficult to discern two consecutive peaks in a chromatogram from each other. Resolution is a function of selectivity of the medium and the efficiency of the medium to produce narrow peak (minimal peak broadening). The resolution ( $R_s$ ) between two peaks in a chromatogram is given by:

$$R_s = 2 \Delta Z / (W_A + W_B)$$

$$= 2 [(t_R)_A - (t_R)_B] / (W_A + W_B) \quad (1)$$

Here in equation 1, the separation between peaks A and B is indicated by  $\Delta Z$ ,  $W_A$  and  $W_B$  are the width at the base of peaks A and B respectively, and  $(t_R)_A$  and  $(t_R)_B$  are the retention time of peak A and B respectively. Acceptable resolution is on the order of  $R_s = 1.0$  and baseline separation between two peaks requires  $R_s > 1.5$ . As mentioned earlier four different types of columns were used to study the resolution of A $\beta$  oligomers and blood serum.

### **Sephacryl S-100:**

The column was equilibrated with the running buffer (20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride). First, 100  $\mu$ l of A $\beta$  oligomers (absorbance at 280 nm = 0.6 AU) were loaded. After the elution of all of the loaded samples at a flow rate of 0.6 ml/min the column was equilibrated again. Then, 50  $\mu$ l of filtered blood serum (absorbance at 280 nm = 43.9 AU) was loaded.

### **Superose 12 HR 10/30**

At first, the column was equilibrated with the running buffer (20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride). Then 25  $\mu$ l of blood serum was loaded with 20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride at a flow rate of 0.5 ml/min. Then, 50  $\mu$ l of A $\beta$ cc oligomers were loaded using the same buffer and same flow rate after equilibrating the column again.

### **Superdex 75 HR 10/300 GL and superdex 200 HR 10/300 GL:**

25  $\mu$ l of blood serum was loaded with 20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride at a flow rate of 0.5 ml/min. Then, 50  $\mu$ l of A $\beta$ cc oligomers were loaded using the same buffer and same flow rate after equilibrating the column again.

### **Optimization of the experimental condition using different flow rates:**

The condition was optimized considering the flow rate in Superdex 200 HR 10/300 (0.25-0.75 ml/min) and the maximum recommended flow rate (1 ml/min).

Different flow rates were applied in Superdex 200 HR 10/300 gel filtration column with A $\beta$  oligomers and blood serum samples separately and the resolution of the separation was observed. First 0.1 ml/min flow rate was applied to the column and the resolution of the

separation was monitored. Similarly, flow rate 0.2 ml/min, 0.3 ml/min, 0.4 ml/min and 0.5 ml/min was applied to Superdex 200 HR 10/300 gel filtration chromatography column and the resolution of separation was monitored.

#### **Sample collection for co-elution study:**

After optimizing the conditions, 50  $\mu$ l of A $\beta$ cc oligomeric solution was loaded on Superdex 200 HR 10/300 column equilibrated with 20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride at 0.5 ml/min flow rate and 0.5 ml fractions were collected. Then 25  $\mu$ l of blood serum was loaded with 20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride at 0.5 ml/min flow rate and 0.5 ml fractions was collected. Fractions after almost 12 ml elution volume were pooled and stored in -20 °C. The stored samples (blood serum fractions and A $\beta$  oligomers) were mixed at a ratio of 1:1, 1:2 and 1:3 respectively at room temperature. After mixing the mix was kept in room temperature for 5, 10, and 15 min respectively before loading on to the gel filtration column.

Another set of samples were prepared on ice. Blood serum fractions and A $\beta$  oligomers were mixed in an eppendorf tube on ice. After preparing the samples of 1:1, 1:2 and 1:3 ratio they were kept on ice for 5, 10 and 10 min respectively before loading on to the gel filtration column.

#### **Co-elution study of A $\beta$ oligomers with blood serum fraction:**

All of the mixtures of different ratios (prepared in room temperature and on ice) of blood serum fractions and A $\beta$  were separately loaded (50  $\mu$ l) on Superdex 200 HR 10/300 column equilibrated with 20 mM sodium phosphate; pH 7.4; 150 mM sodium chloride at 0.5 ml/min flow rate and 0.5 ml fractions were collected.

In the next step of the experiment the fractions that are eluted at the elution volume of A $\beta$  oligomers were analyzed by SDS-PAGE. It was expected that the some binding proteins will also elute with those fractions.

After the SDS-PAGE the gel was silver stained following previously described silver staining procedure.

Some problems raised when working with A $\beta$  in size exclusion chromatography because, oligomers started to stuck to the column after each run. The problem were solved by washing the column using 0.5 M sodium hydroxide after each run.

## Results and discussions:

### Expression:

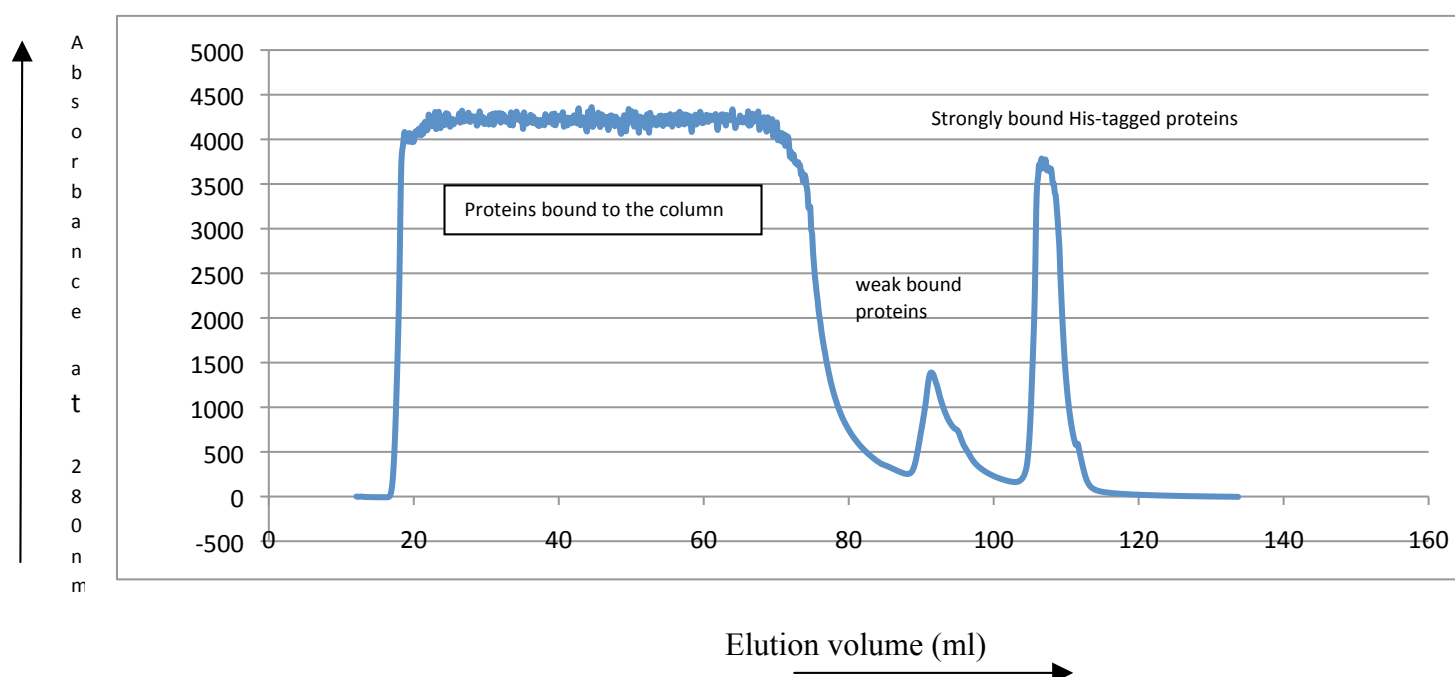
Bacterial cells (E.coli) containing plasmids pACYDuet-1 coding the A $\beta$ 1-42cc were cultured for the expression of A $\beta$ 1-42cc. Table.1 presents the bacterial cell density level before and after the addition of IPTG. After getting the absorption of 0.6 AU then IPTG was added.

**Table.1: Protein expression monitored at OD (600 nm).**

After 1 hour of incubation	After 2 hour of incubation	3 hour of incubation after the addition of IPTG	4 hour of incubation after the addition of IPTG
0.37 AU	0.62 AU	1.14 AU	1.23 AU

### Purification:

After IMAC, fractions supposed to contain A $\beta$ 42cc-Z $A_{\beta 3}$  (3) were collected and concentrated up to abs(280 nm) = 3.9 AU. SEC was run with 2 ml of concentrated IMAC fractions containing A $\beta$ 42cc-Z $A_{\beta 3}$  complex. A $\beta$ 42cc-Z $A_{\beta 3}$  containing fractions were concentrated up to abs(280 nm) = 4.5 AU and stored at -20 °C for denaturing IMAC.

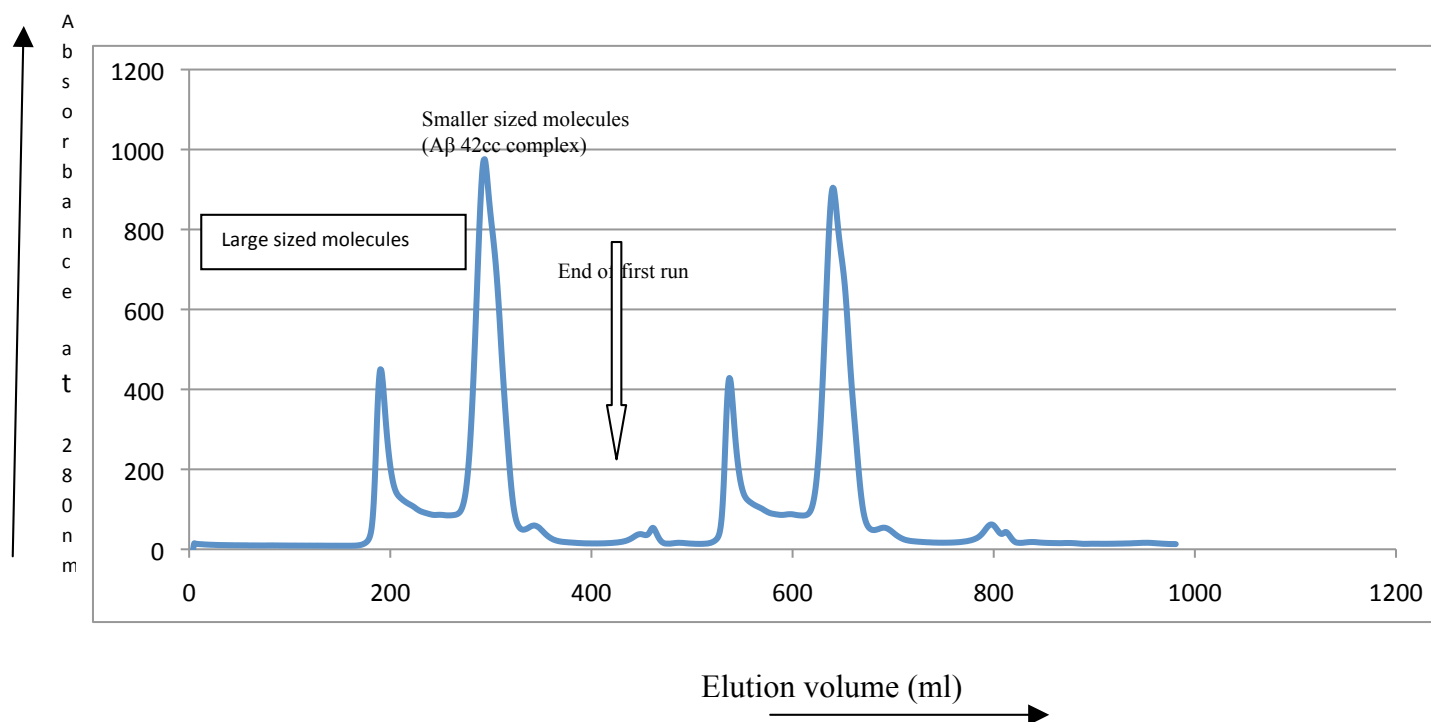


**Fig.3: Immobilized metal ion affinity chromatogram of A $\beta$ 42cc-Z $A_{\beta 3}$  complex.**



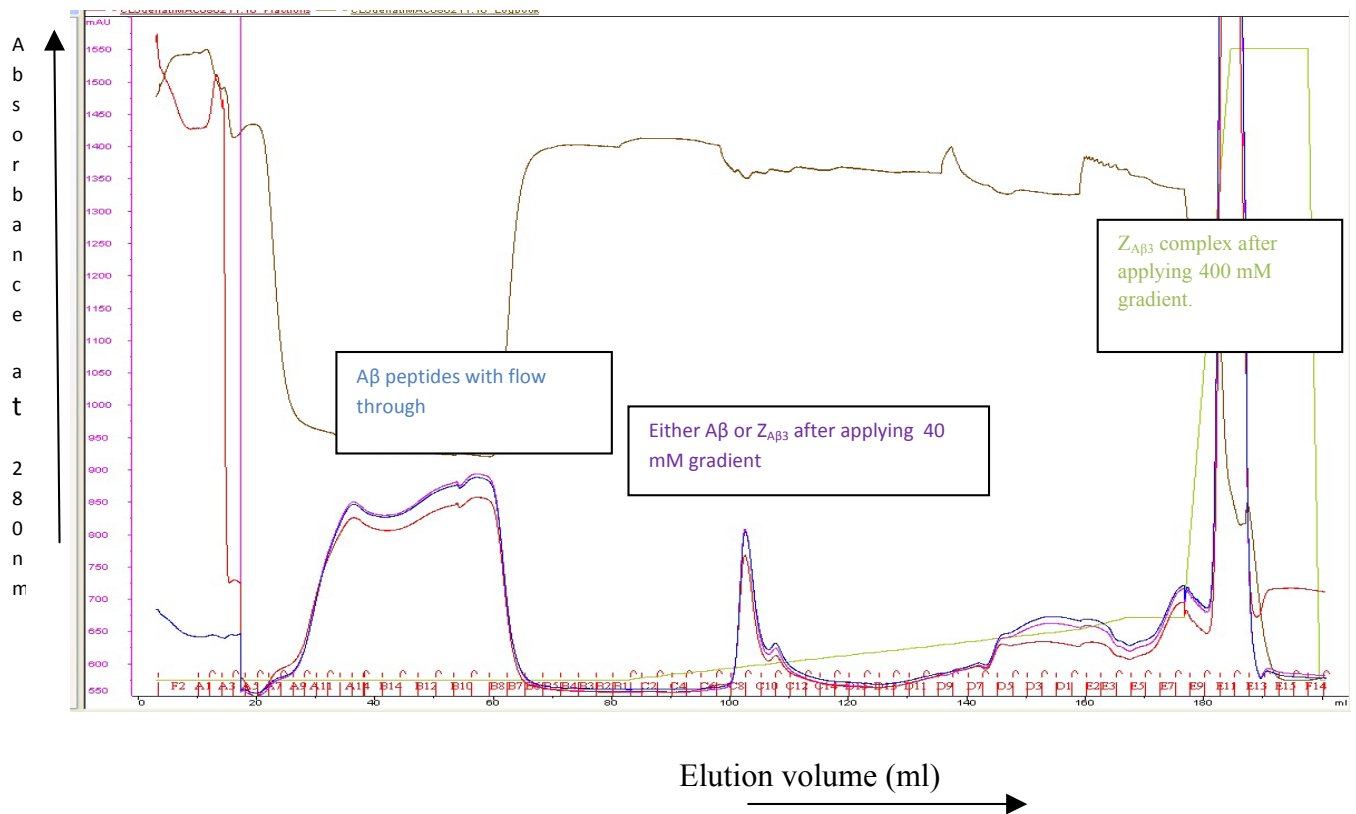
Fig.3 shows the IMAC purification of the complex. The first peak eluted are the proteins that elute with the flow through. In the second peak, proteins that binds weakly to the column are eluted with 4% buffer B. Finally the peak after washing with 100% buffer B indicates the elution of A $\beta$ 42cc-Z $A\beta$ 3 complex.

Fractions which were supposed to contain A $\beta$ 42cc-Z $A\beta$ 3 complex were collected and concentrated up to abs(280 nm) = 3.9 AU.



**Fig.4: Size exclusion chromatogram of A $\beta$ 42cc-Z $A\beta$ 3 complex.**

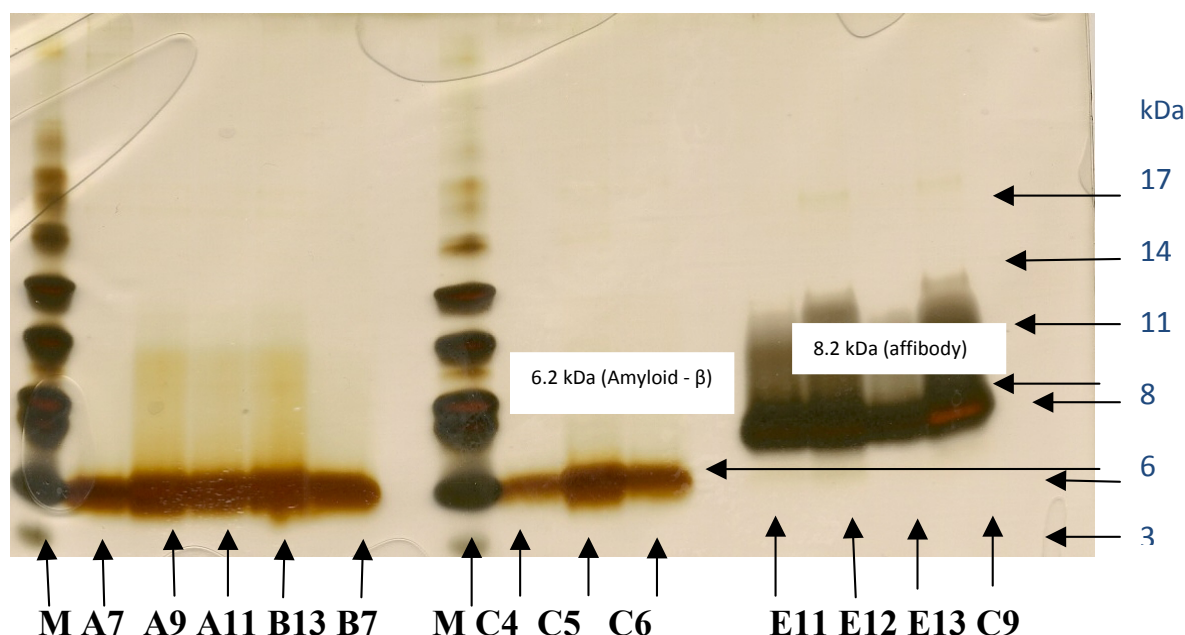
Fig.4 describes two runs of SEC where 2 ml of concentrated samples from IMAC were loaded on each run. Large sized molecules elute first and smaller molecules (i.e. supposed to contain A $\beta$ 42cc-Z $A\beta$ 3 complex) elutes later. Fractions corresponding to large peak were pooled and concentrated using 5 kDa vivaspin cut-off to OD = 4.5 AU at 280 nm.



**Fig.5: Denatured immobilized metal ion affinity chromatogram of 42CC Z<sub>Aβ3</sub> complex.**

The chromatogram in fig.5 represents denatured IMAC. Aβ peptides comes with the flow through of 20 mM tris-hydrochloride pH 8.0; 7 M GdnHCl. The imidazole concentration was increased to 40 mM during a 20 min gradient and as a result additional Aβcc peptide and potentially some Z<sub>Aβ3</sub> were eluted. Finally, when washed by a gradient of 400 mM imidazole, Z<sub>Aβ3</sub> were eluted.

It was observed from the chromatogram that fractions A5-A15, B15-B5 elutes with the flow through (i.e. the fractions supposed to contain Aβcc because Z<sub>Aβ3</sub> is still bound to the column) after washing with a quick gradient up to 400 mM imidazole buffer, bound Z<sub>Aβ3</sub> eluted and the corresponding fractions are E11-E13.



**Fig 6: SDS-PAGE gel diagram showing bands of peptides.**

In fig.6 the SDS-PAGE image showed that, fractions A7, A9, A11, B13 and B7 formed single bands of about 6.2 kDa molecular weight and also the fractions C4, C5 and C6 formed bands of same molecular weight and this indicates that those fractions contained same peptide fractions (A $\beta$ 42cc fractions). Fractions E11-E13 formed bands of higher molecular weight (about 8.2 kDa) than the other fractions. This indicates that those fractions contained affibody (Z<sub>A $\beta$ 3</sub>) peptides.

#### **Production of A $\beta$ 42cc oligomers:**

A $\beta$ 42cc containing fractions from denatured IMAC were concentrated using 3 kDa cut-off to a final concentration of OD 280 nm = 1.3 AU. The sample was dialyzed against 20 mM sodium phosphate pH 7.2; 50 mM sodium chloride with 5 mM EDTA. A $\beta$  oligomers (0.49 mM) formed spontaneously (as the procedure was carried out according to an established protocol) during dialysis.

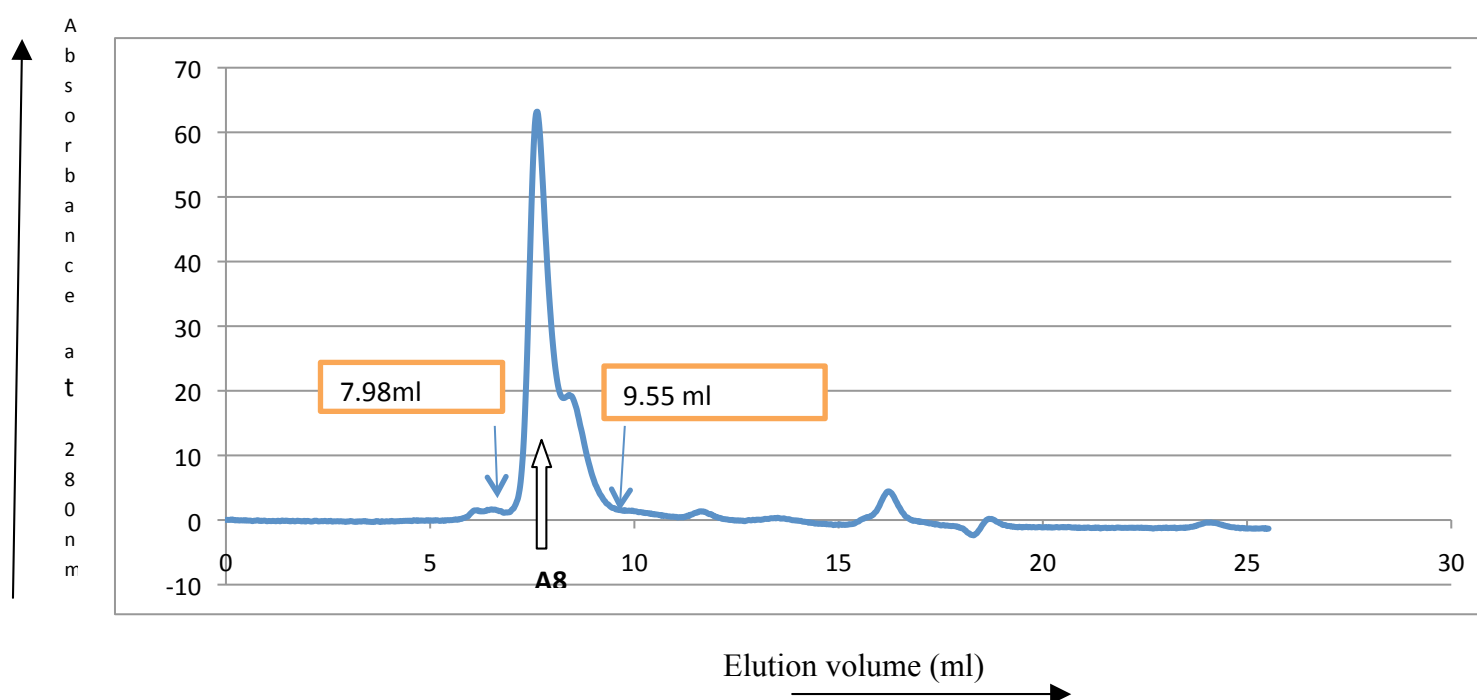
#### **Selection of buffer for size exclusion chromatography of A $\beta$ oligomers and blood serum:**

Buffer of with 20 mM sodium phosphate; pH 7.4; and 50, 100 or 150 mM sodium chloride, were used to test the resolution of separation by loading blood serum and A $\beta$  oligomers on to Superdex 200 10/300. After repeating several times at the same condition the obtained resolution values did not differ much (the resolution after using first buffer was 4.23 and the

resolution after using second buffer was 4.24). The best resolution (4.26) were found using 20 mM sodium phosphate; pH7.4; 150 mM Sodium chloride.

### Selection of gel filtration column with the best resolution:

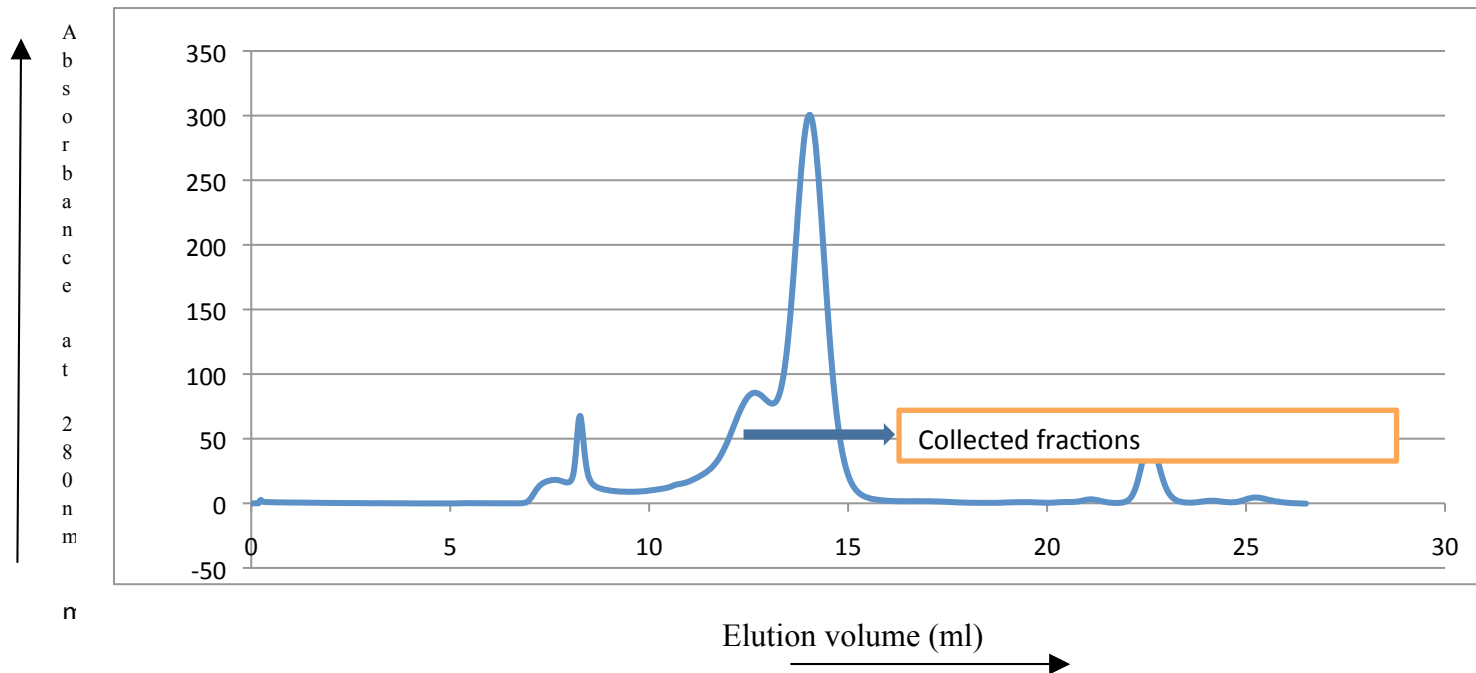
Four different kinds of columns were used to select the best column with better resolution ( $R_s$ ) of separation of A $\beta$  oligomers and blood serum. Sephacryl S-100, Superose 12, Superdex 75 HR 10/300 and Superdex 200 HR 10/300 showed  $R_s$  value 1.76, 1.33, 0.95 and 4.26 respectively. Acceptable resolution is on the order of  $R_s = 1.0$  and baseline separation between two peaks requires  $R_s > 1.5$ . So, Superdex 200 HR 10/300 was the best column with respect to resolution.



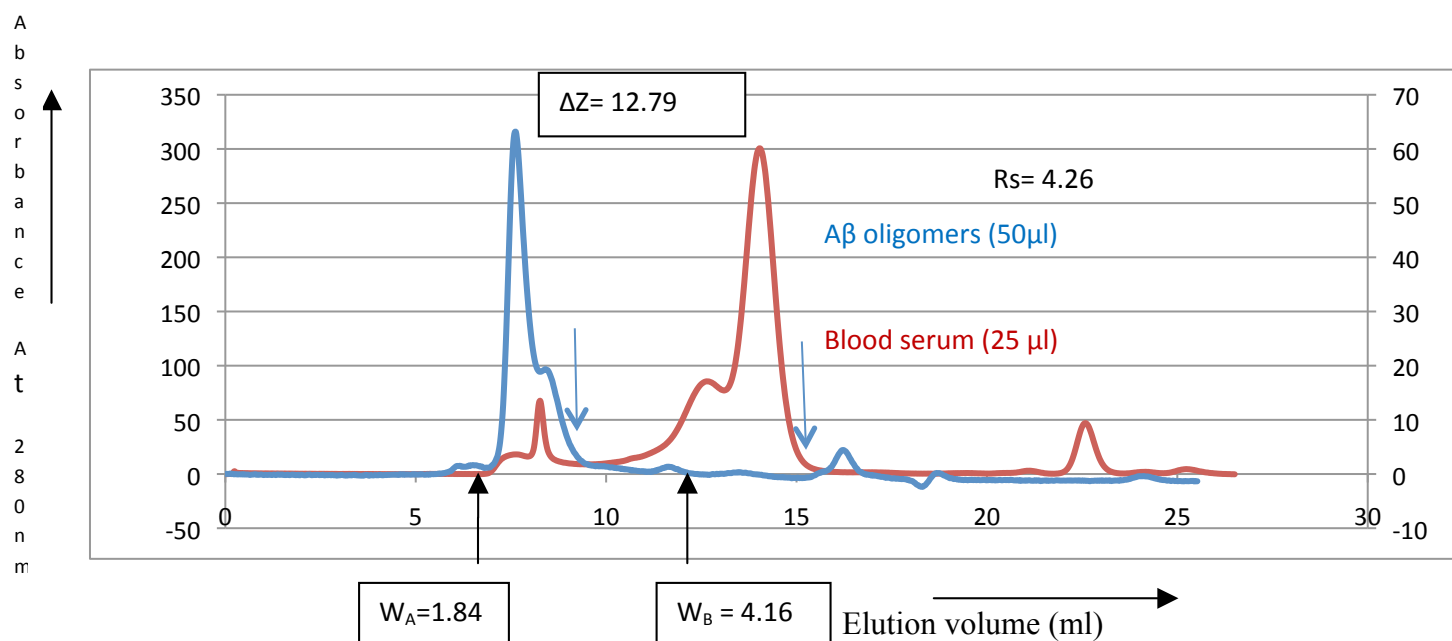
**Fig.7: Size exclusion chromatography diagram showing the separation of A $\beta$  oligomeric sample.** 50  $\mu$ l of sample was loaded on Superdex 200 HR 10/300 GL.

Fig.7 shows that after loading only A $\beta$  oligomers the peak started to rise after 7.98 ml elution volume and started to go down at almost 10 ml of elution volume. After loading only blood serum sample (fig.8) it was observed that at about 7 ml of elution volume one small peak was rising and goes down at 8.12 ml of elution volume. Then another large peak started to rise at 11.2 ml of elution volume and goes down at 15.2 ml of elution volume.

All the fractions after 12 ml of elution volume (no overlap with the peak of A $\beta$ cc oligomers as they have elution volume of about 8 ml) were pooled and stored for further experiments. The protein concentration from the collected fractions was 0.5 AU at 280 nm.



**Fig.8: Size exclusion chromatography diagram showing the separation of blood serum sample. 25  $\mu$ l of sample was loaded on Superdex 200 HR 10/300.**



**Fig 9: Size exclusion chromatogram of A $\beta$  oligomers and blood serum at 280 nm in Superdex 200 HR 10/300. Superdex 200 HR 10/30 showed  $R_s$  value 4.26 at 0.5 ml/min flow rate.**

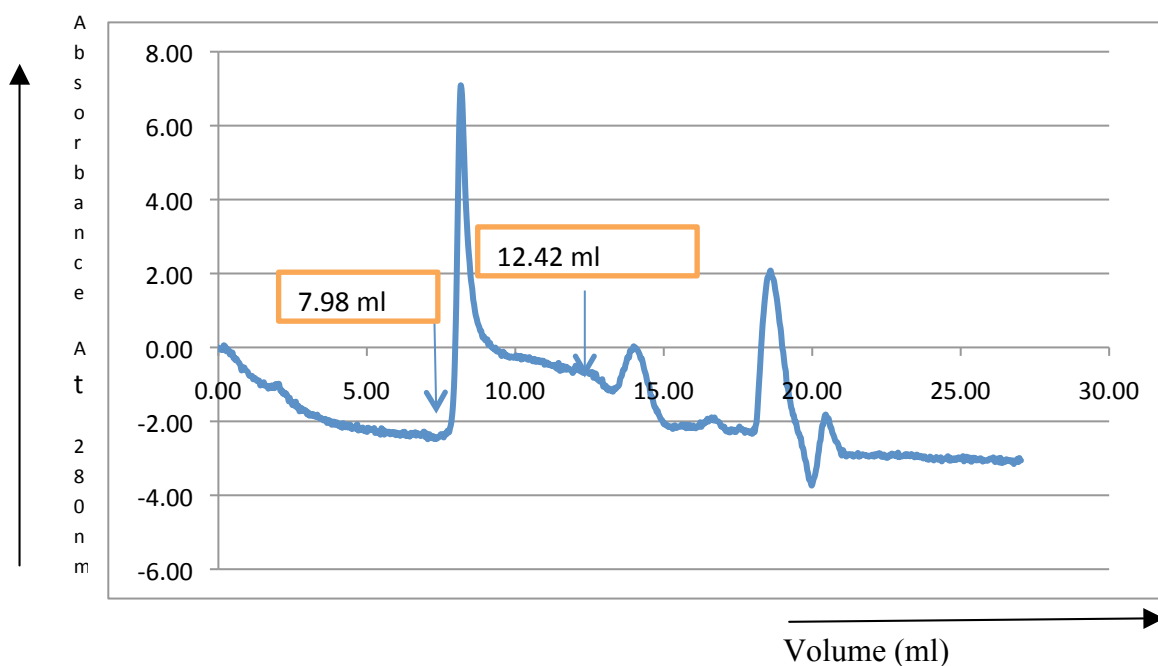
In fig.9 the resolution was calculated from equation.1, where the separation between peaks A and B is indicated by  $\Delta Z$ , retention time of peak A ( $t_{RA}$ ) is 27.95 ml, retention time of peak B ( $t_{RB}$ ) is 15.52 ml and width of peak A ( $W_A$ ) and B ( $W_B$ ) are 1.84 ml and 4.16 respectively.

### **Optimization of the experimental condition using different flow rates:**

As part of optimization of conditions for SEC separation of A $\beta$  and blood serum, I have tried with different flow rates to find the best resolution. I have applied the flow rates 0.1 ml/min, 0.2 ml/min, 0.3 ml/min, 0.4 ml/min and 0.5 ml/min, in the experiments using Superdex 200 gel filtration column with 20 mM sodium phosphate; 150 mM sodium chloride; pH 7.4 as running buffer using both of A $\beta$  and blood serum separately and found  $R_s$  value 2.54, 2.39, 2.19, 3.44 and 4.26 respectively. Among all of the flow rates, 0.5 ml/min showed the best resolution (4.26) for the separation.

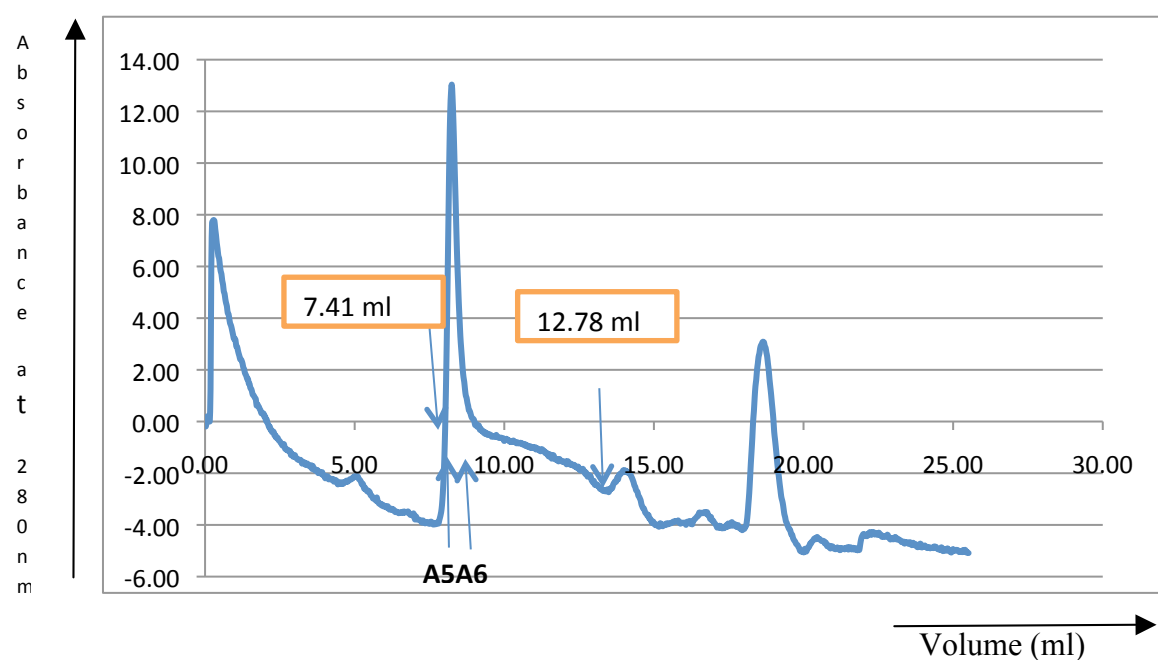
A $\beta$  oligomers and blood serum fractions (after 12 ml of elution volume, see above) that are collected after loading 25  $\mu$ l of blood serum were mixed in ratios of 1:1, 2:1, and 3:1 respectively in room temperature and also on ice. All samples with different conditions (room temperature or on ice, mixing time) showed almost the same results (shown later with chromatograms fig 10-12).

When loading the samples the concentration of A $\beta$  oligomeric fraction was 0.6 AU (absorbance at 280 nm), the concentration of blood serum sample was 43.9 AU as measured by absorbance at 280 nm.



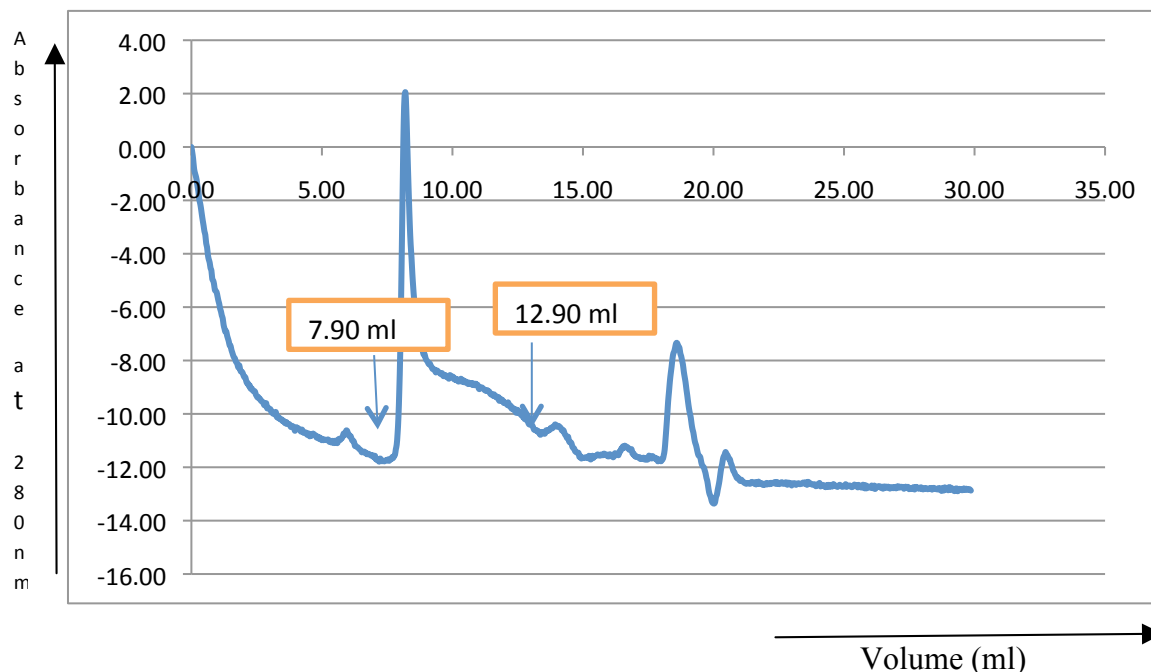
**Fig 10: Elution profile of A $\beta$  oligomers and blood serum fractions (1:1) observed at 280 nm in Superdex 200 gel filtration column.** Sample was mixed at room temperature and kept 5 min prior loading the sample.

In fig.10, it was observed that A $\beta$  oligomeric fractions were started to elute at 7.98 ml as the elution peak started to rise and the peak goes down at 12.42 ml of elution volume. The corresponding fractions were collected for further analysis.



**Fig 11: Gel filtration chromatogram of A $\beta$  oligomers and blood serum fractions (ratio 2:1) observed at 280 nm.**

Sample shown in fig. 11 was mixed on ice and kept 10 min on ice prior loading the sample. A $\beta$  oligomeric fractions were started to elute at 7.41 ml of elution volume and peak goes down at 12.78 ml (shown in fig.11). The corresponding fractions were analyzed by SDS-PAGE.



**Fig 12: Gel filtration chromatogram of A $\beta$  oligomers and blood serum fractions (3:1) observed at 280 nm.**

Sample shown in fig. 12 was mixed on ice and kept 15 min on ice prior loading the sample. From fig.12, it was observed that A $\beta$  oligomeric fractions were started to elute at 7.90 ml of elution volume and absorbance peak goes down at 12.90 ml of elution volume.

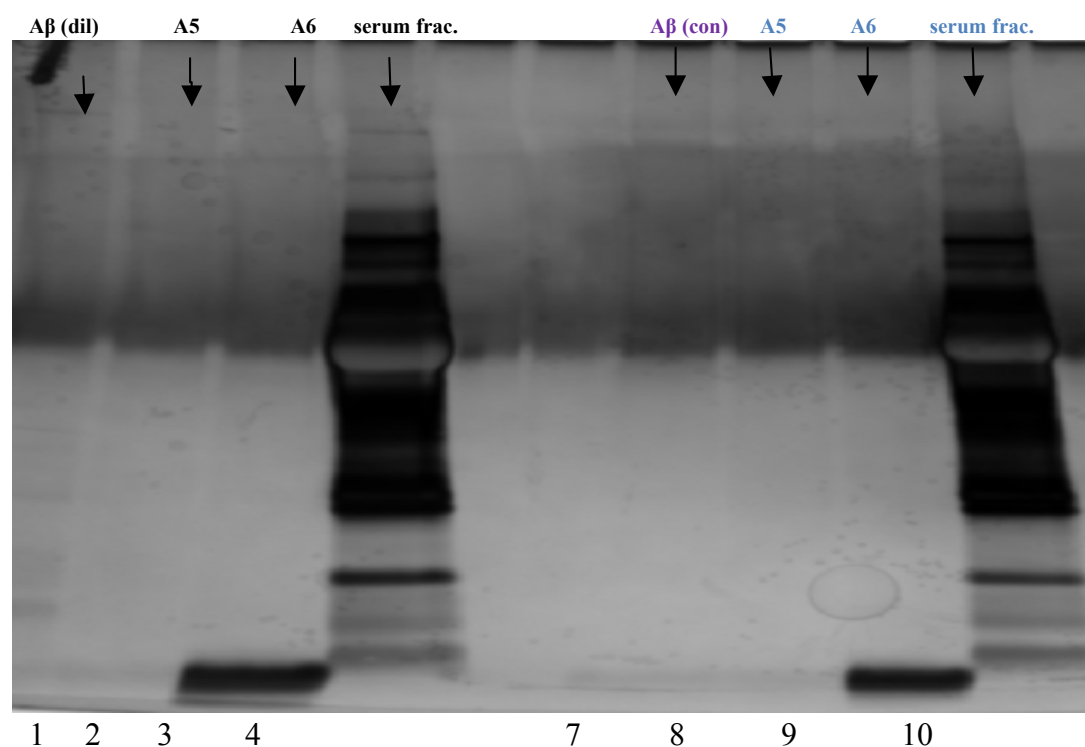
All the fractions that were eluted at the same elution volume as the A $\beta$ cc oligomers were loaded on SDS-PAGE. Because it was expected that A $\beta$  oligomers will elute with the binding proteins. It was observed on gel that only A $\beta$  monomeric, dimeric, trimeric and a weak band of blood serum protein was observed.

The concentrations of the samples before loading on gel (shown in fig.13 and 14) are listed in the table 2.



**Table 2: Protein concentration of samples before loading on the gel.**

Sample	A $\beta$ fraction diluted (A8)	A $\beta$ +serum Diluted (A5)	AB+serum diluted (A6)	Serum fractions diluted	A $\beta$ fraction conc. (A8)	A $\beta$ +serum conc. (A5)	A $\beta$ +serum conc. (A6)	serum conc.
Absorption at 280 nm (AU)	0.022	0.018	0.016	0.5	0.03	0.023	0.021	1.8

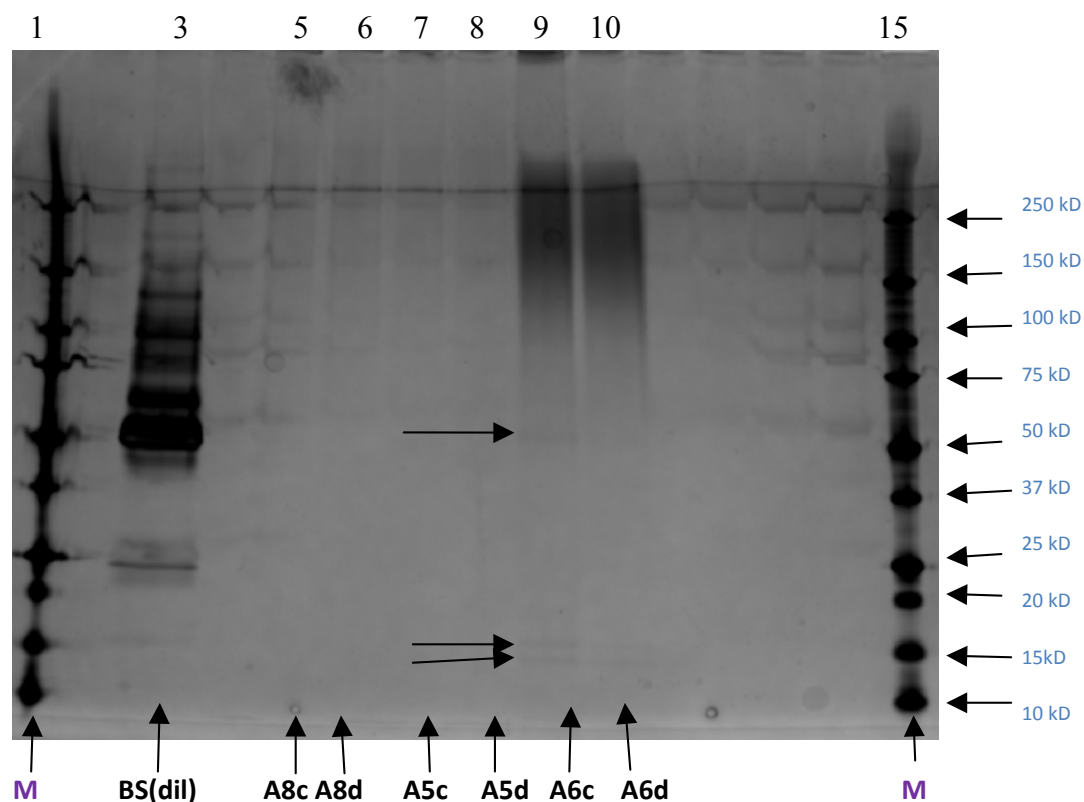


**Fig.13: SDS-PAGE gel diagram showing bands of peptides after silver staining (without using molecular size marker).** Lane 1: diluted A $\beta$  oligomeric fraction that was collected at a elution volume of about 8 ml after loading only A $\beta$  oligomers (chromatogram shown in fig.7); Lane 2: fraction A5; Lane 3: fraction A6, fractions that were collected after loading mix of A $\beta$  oligomers and blood serum fraction (chromatogram shown in fig.11); Lane 4: blood serum fractions that were collected after loading only blood serum at a elution volume of 12 ml (chromatogram shown in fig.8) were loaded respectively. On lane 7, 8, 9 and 10 same fractions after concentration were loaded.

From fig. 13 it was observed that on lane 1 and 2 (left) weak A $\beta$ cc monomeric bands appeared from the A $\beta$ cc with blood serum (1:2) diluted fraction. On lane 3, strong A $\beta$ cc monomeric band was appeared from A $\beta$ cc with blood serum (1:2) diluted fraction. No other bands of high molecular weight molecules were observed. That means no molecules from

blood serum fractions were strongly interacting with A $\beta$ cc oligomers. On lane 4, Blood serum fractions showed different bands. Corresponding A $\beta$ cc fraction (lane 1) and blood serum fractions (lane 4) that were collected after 12 ml of elution volume were loaded as control. No molecular size marker was loaded.

In another gel run molecular size marker was used to analyze the fractions. Blood serum fractions (chromatogram shown in fig.8) were diluted 1:10. Then the other fractions like A8 (chromatogram shown in fig.7), A5 and A6 (chromatogram shown in fig.11) were loaded both in concentrated and diluted form. A8 fraction supposed to contain A $\beta$  after loading only A $\beta$  oligomers. A5 and A6 fractions were collected at the same elution volume of only A $\beta$  oligomeric fractions after mixing with blood serum fractions at 2:1 ratio.



**Fig.14: SDS-PAGE gel diagram showing bands of different peptides.** On lane 1 and 15, 2  $\mu$ l of 1:10 diluted molecular size marker was loaded. On lane 3 diluted blood serum sample was loaded. On lane 5 and 6 concentrated and diluted A $\beta$  fractions (collected after loading only 50  $\mu$ l of A $\beta$  oligomers), on lane 7, 8, 9 and 10 concentrated and diluted fractions from 1:2 (blood serum: A $\beta$ cc oligomers) mix were loaded respectively.

From fig .14, it was observed that, on lane 9 (concentrated A6 fraction) between 50 kDa and 75 kDa there have one weak band and this could be a binding protein from blood serum. It

might be immunoglobulin (immunoglobulin consist of two light and two heavy chains and the molecular weight of heavy chains 51-72 kDa). It seems to correspond to the strongest band in the serum sample. The other two bands between 14 to 17 kDa are the dimer and trimer bands of A $\beta$ cc(22).

The "smeary" bands between 100-250 kDa in the A6 samples might be A $\beta$ 42cc aggregates. This would be in agreement with the shift from monomeric A $\beta$ 42cc on the other gel (fig 13) to the dimers/trimers seen on this gel. So, from the above experiments we could not observe any strong interaction between A $\beta$ cc oligomers and blood serum proteins.

## Conclusion:

In my project, I have tried to develop a method to study the interaction of proteins of human blood serum with neurotoxic A $\beta$  oligomers. I have introduced SEC as a part of method development but no interaction was observed. The storage condition of A $\beta$ cc may be one reason as we observed monomeric, dimeric and trimeric bands of A $\beta$ cc on gel. Another reason may be that there was not sufficient amounts of blood serum proteins present that could bind with A $\beta$ . The obtained result suggests that therapeutics based on protein ligand for Alzheimer's disease research needs to introduce other methods. However, SEC is an excellent technique to separate the molecules according to their sizes. If the molecules interacted then it would be easier to analyze by further experiments like SDS-PAGE and mass spectrometry. Moreover, SEC is reliable, easy to handle and not so time consuming technique. In our experiment we tried to use different conditions to allow interaction of A $\beta$  and blood serum proteins. The general lessons from our condition optimization experiments are that A $\beta$  oligomers and blood serum showed the better resolution at a medium flow rate although some literature suggested that lower flow rate gives the higher resolution. The reason could be the oligomeric nature of A $\beta$ . As no strong interaction was observed, further development of the technique using wide range of column designs, lengths, blood serum proteins with proper concentration, proper storage and mixing condition of A $\beta$  oligomers with blood serum proteins is expected to contribute to our understanding of the A $\beta$  oligomers-blood serum protein interaction.

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